BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 January 2001 (25.01.2001)

PCT

(10) International Publication Number
WO 01/05397 A1

(51) International Patent Classification[7]: A61K 31/35

(21) International Application Number: PCT/KR00/00769

(22) International Filing Date: 14 July 2000 (14.07.2000)

(25) Filing Language: Korean

(26) Publication Language: English

(30) Priority Data:
1999/28877 16 July 1999 (16.07.1999) KR

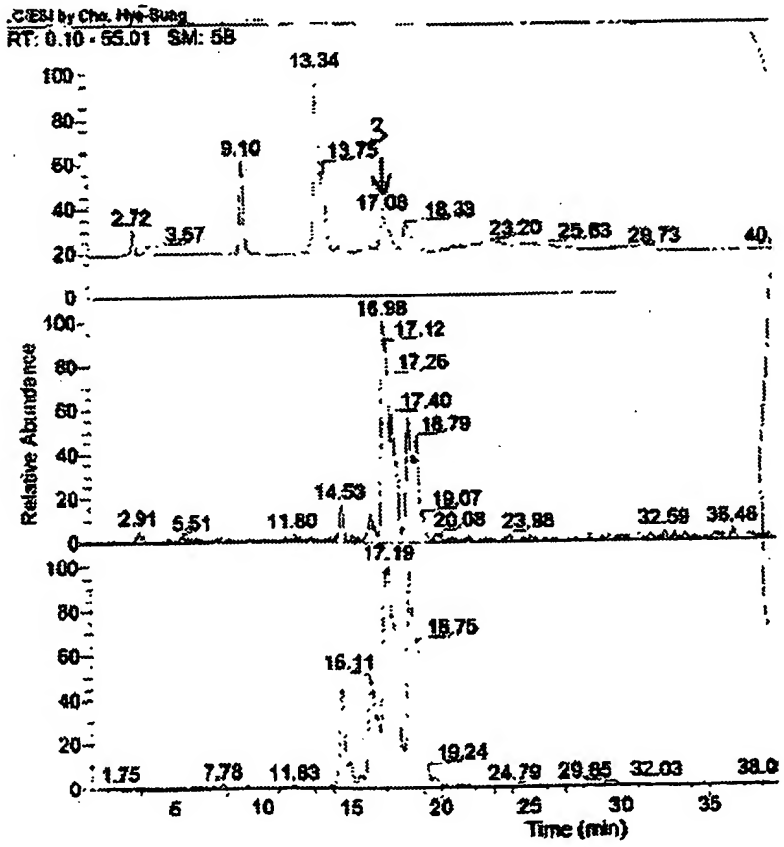
(71) Applicant *(for all designated States except US)*: **LG CHEMICAL LTD.** [KR/KR]; LG Twin Tower, Yoido-dong 20, Yongdungpo-ku, Seoul 150-721 (KR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **KIM, Sang-Nyun** [KR/KR]; Sejong Apt. 106-102, Jeonmin-dong 462-5, Yuseong-ku, Taejeon-city 305-728 (KR). **AHN, Ho-Jeong** [KR/KR]; Sejong Apt. 107-1106, Jeonmin-dong 460-5, Yuseong-ku, Taejeon-city 305-728 (KR). **PARK, Sang-Ki** [KR/KR]; LG Sataek 2-404, Doryong-dong 386-4, Yuseong-ku, Taejeon-city 305-340 (KR). **KIM, Moon-Moo** [KR/KR]; Lucky Apt. 1-202, Doryong-dong 386-4, Yuseong-ku, Taejeon-city 305-340 (KR). **KIM, Jung-Hun** [KR/KR]; LG Apt. 1-103, Doryong-dong, Yuseong-ku, Taejeon-city 305-340 (KR). **LEE, Hak-Mo** [KR/KR]; LG Apt. 2-404, Doryong-dong, Yuseong-ku, Taejeon-city 305-340 (KR). **PARK, Hyoung-Kook** [KR/KR]; Cheongsol Apt. 512-705, Songgang-dong 8-2, Yuseong-ku, Taejeon-city 305-752 (KR). **CHO, Hye-Sung** [KR/KR]; Hanvit Apt. 110-1401, Eoeun-dong 99, Yuseong-ku, Taejeon-city 305-755 (KR). **JEOUNG, Seung-Young** [KR/KR]; Kolon Apt. 6-502, Nae-dong 151, Seo-ku, Taejeon-city 302-727 (KR).

[Continued on next page]

(54) Title: PROCYANIDIN OLIGOMERS FOR INHIBITING MATRIX METALLOPROTEINASES AND MEDICINE HAVING EFFECTIVE COMPOSITION OF SAME



(57) **Abstract:** The present invention provides procyanidin oligomers with significant biological activity against matrix metalloproteinase (MMP). The procyanidin oligomers can be isolated from the genus Ulmus and other plants and comprise trimeric through dodecameric procyanidin oligomers of flavan-3-ol monomer units. The present invention encompasses methods of using the procyanidin oligomer in treating tumor metastasis or invasion, rheumatoid arthritis, diabetes, corneal, epidermal, and gastric ulceration, skin wrinkling, periodontitis, osteoporosis; and in the promotion of wound and burn healing and other related maladies in which uncontrolled high levels of MMP are thought to play an important role in the malady progress.

WO 01/05397 A1

(74) **Agent: KIM, Won-Ho**; 7th Fl., Teheran Bd., 825-33, Yoksam-dong, Kangnam-ku, Seoul 135-080 (KR).

(81) **Designated States** *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

PROCYANIDIN OLIGOMERS INHIBITING MATRIX METALLOPROTEINASES AND MEDICINE HAVING EFFECTIVE COMPOSITION OF THE SAME

CROSS REFERENCE TO RELATED APPLICATION

This application is based on application No. 10-1999-0028877 filed in the Korean Industrial Property Office on July 16, 1999, the content of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The present invention relates to procyanidin oligomers, more particularly to procyanidin oligomers inhibiting activities of matrix metalloproteinase (hereinafter referred to as MMP) which decomposes an extracellular matrix and basement membrane of connective tissues. These procyanidin oligomers can be obtained from *Ulmus* cortex of the genus *Ulmus* and other plants, and is composed of 3 to 12 of basic units, flavan-3-ol. These procyanidin oligomers can be used as a medicine for preventing and treating metastasis of cancer, paradental disease, rheumatoid arthritis, diabetes, corneal ulcer, epidermal ulcer, gastric ulcer, wrinkles and aging of skin, paradentitis, osteoporosis, injury, burn, and related diseases in which MMP plays an important role.

(b) Description of the Related Art

Matrix metalloproteinase (MMP) is a calcium and zinc-dependent

endopeptidase which is secreted from cells such as polymorphonuclear neutrophil, macrophage, fibroblast, and bone cells, acting at neutral pH, and it uses various extracellular matrixes as its matrix. This matrix metalloproteinase is known to be involved not only in numerous physiological processes such as embryogenesis, tissue formation, salivary gland formation, and teething, but also in pathological processes and various diseases such as wound, metastasis of cancer, paradental disease, rheumatoid arthritis, inflammation, diabetes, corneal ulcer, osteoporosis, gastric ulcer, trauma, wrinkling and aging of skin, and wound and burn healing.

Particularly, type IV collagenases MMP (MMP 2 and MMP 9), i.e., 72-kD and 92-kD collagenases are the most important enzymes in the infiltration and metastasis of cancer cells, because they decompose type IV collagen which is a main structural constituent of the basement membrane that is the first barrier to metastasis of cancer. In paradental disease, MMP causes collagenases secreted from fibroblast, polymorphonuclear leukocytes, epithelia, and macrophage and collagenases secreted from paradental bacteria to decompose collagen that is the matrix of paradentium, thereby forming gingival recessions, which is proceeded to paradental diseases if continuously stood. Furthermore, it has been recently found that MMP is very closely concerned with aging of skin, aging of skin due to light, and wrinkle formation (Br. J. Dermatol., 2000; 142, 267-273, Arch. Dermatol. Res., 2000; 292, 27-31, Free Radical Biol. Med., 1999; 27, 729-737).

Therefore, collagenase inhibitors function as a medicine which is useful in preventing and treating infiltration and metastasis of cancer, and

diseases resulting from the decomposition of collagenic connective tissues, such as paradental disease, rheumatoid arthritis, inflammation, skin wrinkling and aging, diabetes, corneal ulcer, epidermal ulcer, gastric ulcer, osteoporosis, trauma and burn, infiltration and metastasis of cancer, and collagenic connective tissue decomposition.

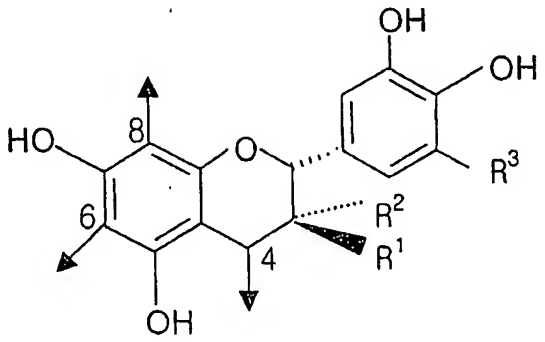
Medicines so far found that can inhibit activities of MMP include tetracyclines such as tetracycline, minocycline and doxycycline, and peptide derivatives.

The peptide derivatives are similar to collagens, and enzyme inhibitors comprising hydroxamic acid, thiol, and carboxylalkyl groups being capable of chelating zinc ions in active portion of a collagenase enzyme has been actively studied (Pharmacol. Ther., 1997; 75, 69-75, U.S. Patent Nos. 4,996,358, 5,183,900, 5,300,674, 5,861,436, etc.). For example, it has been disclosed in U.S. Patent No. 5,514,677 that rheumatoid arthritis, inflammation, skin disease, osteolysis disease, metastasis of cancer, and wounds can be treated through collagenase inhibition using hydroxamic acid. Furthermore, it is disclosed in U.S. Patent No. 4,666,897 that excessive collagenase activities can be inhibited using tetracycline, minocycline, and doxycycline.

A procyanidin represented by the following Formula 1 is a common designation of oligomers and polymers of dimmer or more having a backbone of catechin, epicatechin, catechin gallate, epicatechin gallate, gallocatechin gallate, or epigallocatechin gallate, and it is a non-hydrolytic tannin contained in a wide range of plant bodies. Such procyanidin is known to have a capacity of binding to protein, and particularly dimers are reported to have

anti-inflammatory efficacies. And, catechin, which is known to be a green tea tannin, is reported to have excellent antitumor effects besides.

[Formula 1]



Wherein when the procyanidin is catechin R^1 is OH, and R^2 and R^3 are H; when it is epicatechin R^1 is H, R^2 is OH, and R^3 is H; H when it is epicatechin-3-O-gallate R^1 is H, R^2 is O-galloyl, and R^3 is; when it is epigallocatechin R^1 is H, and R^2 and R^3 are OH; and when it is epigallocatechin-3-O-gallate R^1 is H, R^2 is O-galloyl, and R^3 is OH. Furthermore, polymers are formed through bonds between monomers (4-8 or 4-6).

Technologies relating to antioxidation effects and antiviruses, bacterial adherence preventing effects of procyanidin are disclosed in U.S. Patent Nos. 5,494,661, 5,877,206, 5,646,178, 4,797,421, and International Patent Publication No. WO 93/24106, etc. In addition, technologies relating to pharmaceuticals using procyanidin (European Patent Publication No. 812592, Japanese Patent Laid-open Publication No. Sho 61-83958; etc.), cosmetics using procyanidin (European Patent Publication No. 694305, Japanese Patent Laid-open Publication No. Sho 63-36420, etc.), food additives using procyanidin (Japanese Patent Laid-open Publication Nos. Hei

10-004923, Hei 7-49333, etc.) and methods of preparation of procyanidin (Japanese Patent Laid-open Publication No. Sho 63-267774, South African Patent Publication No. 8205023, etc.) are disclosed.

Furthermore, various research results with regard to inhibition of matrix metalloproteinase by green tea ingredients have been reported. For examples, the green tea ingredients, epicatechin gallate and epigallocatechin gallate, inhibit activities of microorganisms and collagenases in gingival crevicular fluid (J. Periodontal., 1993; 64, 630-636); epicatechin gallate, epigallocatechin gallate and theaflavin inhibit infiltration of cancer cell line into gelatin membrane (J. Agric. Food. Chem. 1999; 47, 2350-2354); effects of epicatechin gallate and epigallocatechin gallate of green tea on MMP-2, MMP-9 and MMP-12 have been shown (Biochem. Biophys. Acta, 2000; 1478, 51-60); catechin and theaflavin from green and black tea have shown activities of inhibiting MMP-2 and MMP-9 of lung cancer cell line (Biosci. Biotech Biochem., 1997; 61, 1504-1506), etc. However, all of these disclosures relate to catechin or catechin gallate derivatives having low molecular weights, and nothing is known about inhibition of MMP emzyme by procyanidin oligomer mixture of the present invention in which 3 to 12 of flavan-3-ol's are polymerically connected.

On the other hand, *Ulmus* cortex, which means the barks of root and stem of *Ulmus macrocarpa, Pumila, Davidiana, Americana* of the genus *Ulmus* have traditionally been used for inflammation, gastric ulcer, etc. Recently, U.S. Patent No. 6,045,800 reported that *Ulmus* cortex has excellent activity of inhibiting collagenase relating to paradentitis, and results

relating to the evaluation of inflammatory inhibition thereof have been announced (J. Ethanopharm., 1998; 62, 129-135).

SUMMARY OF THE INVENTION

As a result of studies for investigating which ingredient of *Ulmus* cortex has excellent collagenase inhibiting activity, the present inventors found that the procyanidin oligomer is a main ingredient.

Therefore, it is an object of the present invention to provide a procyanidin oligomer that inhibits the activities of matrix metalloproteinase (MMP).

It is another object of the present invention to provide a pharmaceutical composition comprising a natural ingredient, procyanidin oligomer, which is superior to and safer than a synthesized matrix metalloproteinase inhibitors, such as conventional doxycycline, etc., and natural epigallocatechin gallates, as an active ingredient.

BRIEF DESCRIPTION OF THE DRAWINGS

A more complete appreciation of the invention, and many of the attendant advantages thereof, will be readily apparent as the same becomes better understood by reference to the following detailed description when considered in conjunction with the accompanying drawings, wherein:

Fig. 1 shows the results of analyzing silica ethylacetate fractions using HPLC(high performance liquid chromatography)/ESI(electron spray ionization) mass spectrometer, Finnigan LCQ, indicating the presence of a procyanidin monomer having a molecular weight of 290 (9.1 minutes), two kinds of procyanidin dimers having a molecular weight of 578 (17.08 minutes,

18.33 minutes), and unidentified materials having a molecular weight of 422 (13.34 minutes, 13.75 minutes);

Fig. 2 shows the results of detecting [M + sodium]$^+$ masses in which sodium is added to the molecular weight of main ingredient of fraction 4, procyanidin oligomers, using cyano-4-hydroxycinnamic acid as a matrix, by MALDI-TOF (Matrix-assisted laser desorption/ionization time-of-flight);

Fig. 3 shows the results of tracing [M – H]$^-$ mass of 1441 and [M + TFA$^-$]$^-$ mass of 1555 of pentamers, using HPLC/ESI mass spectrometer;

Fig. 4 shows the results of HPLC/ESI mass spectrometer, indicating that procyanidin oligomer peaks predicted in trimers to dodecamers are detected at fraction 4, and that the ion distribution having predicted masses is transferred from an early stage to a later stage of the chromatogram of fraction 4 as the degree of polymerization are increased from 3;

Fig. 5 shows the results of detecting mass peaks in a scan mode at a retention time zone on a chromatogram near designated mass peaks using HPLC/ESI mass spectrometer, indicating that the main ingredient of fraction 4 is procyanidin oligomer since the main mass peaks are 1153 ([M – H] $^-$) and 1267 ([M + TFA$^-$] $^-$) in case the retention time range was set for tetramers, and they are 1441 ([M – H] $^-$) and 1555 ([M + TFA$^-$]$^-$) in case the retention time range was set for pentamers; and

Fig. 6 shows the results of comparing inhibitory effects of procyanidin oligomer with those of doxycycline on type IV collagenase MMP secreted from peridontal ligament cells by zymography, indicating that the inhibitory effects of procyanidin oligomer are approximately 10 times superior

to those of doxycycline. In Fig. 6, reference numeral 1 is the effects of procyanidin oligomer, and reference numeral 2 is those of doxycycline.

DETAILED DESCRIPTION OF THE INVENTION

In the following detailed description, only the preferred embodiments of the invention have been shown and described, simply by way of illustration of the best mode contemplated by the inventor(s) of carrying out the invention. As will be realized, the invention is capable of modification in various obvious respects, all without departing from the invention. Accordingly, the description is to be regarded as illustrative in nature, and not restrictive.

In order to achieve the objects as described in the above, the present invention provides a procyanidin oligomer that inhibits the activities of matrix metalloproteinase (MMP).

The present invention also provides a pharmaceutical composition for preventing and/or treating diseases resulting from the activities of matrix metalloproteinase (MMP), comprising the procyanidin oligomer as an active ingredient.

The present invention will now be explained in more detail.

The present inventors have studied the active ingredients of *Ulmus* cortex disclosed in U.S. Patent No. 6,045,800 which is traditionally known to be effective on wounds, metastasis of cancer, paradental disease, rheumatoid arthritis, inflammation, corneal ulcers, osteoporosis, gastric ulcers, trauma, wrinkles, acne, burns etc. in order to find an MMP activity inhibitor which is safe and has excellent efficacies in human bodies. As a result,

the present inventors identified that a procyanidin oligomer mixture in which 3 to 12 flavan-3-ol basic units are polymerically connected is a main active ingredient of *Ulmus* cortex, and its inhibitory effects against MMP are superior to those of conventional doxycycline or epigallocatechin gallate, and completed the present invention.

The procyanidin oligomer is preferably used as an MMP activity inhibitor. The MMP is selected from the group consisting of collagenolytic protease (from Kamchatka crabs, purchased from Sigma Corporation), MMP-1, MMP-8, and type IV collagenase of MMP-2 and MMP-9.

The procyanidin oligomer is preferably prepared in the form of a tablet, capsule, powder, ointment, solution, gel, paste, patch, granule, etc., and the contents of procyanidin oligomer contained in the preparation is preferably 0.0001 to 5 wt%.

The procyanidin is separated from n-butanol fractions obtained when a primary extracts that are extracted from the *Ulmus* cortex of the genus *Ulmus* with a polar solvent are solvent-fractionated with n-hexane, dichloromethane, ethylacetate and n-butanol. When Sephadex LH-20 column chromatography is conducted on the n-butanol fractions, the fractions are separated such that the procyanidin oligomer is concentrated. For example, the n-butanol fractions are eluted with water-methanol mixture in its increased order to 80% to 100% methanol, or by different method, sequentially eluted by 100% methanol into various fractions, and the procyanidin oligomer is concentrated by recombination based on thin layer chromatography.

The procyanidin oligomer, a mixture of trimers to dodecamers in which 3 to 12 flavan-3-ol basic units are connected, has a molecular weight of 1,518, an average degree of polymerization of 5.3, and it can be extracted from the group consisting of grapestone, rhubarb, polygoni multiflori radix, camphor tree, cinnamon bark, Chinese arborvitae, camellia seeds, kaoliang, buckwheat, and oak trees which contain much of the same ingredients as *Ulmus* cortex, as well as from *Ulmus* cortex.

The present invention will be explained in more detail with reference to the following Experiments, Examples and Comparative Examples. However, these are only to illustrate the present invention and the present invention is not limited thereto.

[Experiment]

Experiment 1: separation of an active ingredient procyanidin oligomer and identification of the structure thereof

A procyanidin oligomer was separated from an *Ulmus* cortex extract and the structure thereof was identified. However, procyanidins can also be extracted and purified from plants such as grapestone, rhubarb, polygoni multiflori radix, camphor tree, cinnamon bark, Chinese arborvitae, camellia seeds, kaoliang, buckwheat, oak trees, etc. since they are generally known to be contained in large quantities in plants.

Experiment 1-1: crude extract preparation and collagenase activity inhibition

A primary extract was obtained by pulverizing *Ulmus* cortex to a size of 10 to 200 mesh, adding an extract solvent to the pulverized plant powder, cold immersing the mixture at room temperature for 72 hours, filtering the

resultant, and concentrating the filtered extract. The extract solvents are preferably selected from the group consisting of purified water, methanol, ethanol, propanol, butanol, glycerol, ethylene glycol, propylene glycol, 1,3-butylene glycol, ethyl acetate, acetone, and a mixture thereof.

Fractions were obtained by suspending the obtained primary extract in water and then sequentially solvent-fractionating the suspended extract using n-hexane, dichloromethane, ethylacetate, and n-butanol. The remaining filtrate after the solvent-fractionating was taken as a water fraction. An inhibition of the activities of collagenase that is one kind of MMP was tested using the obtained 5 kinds of solvent fractions.

As results, the n-hexane or dichloromethane fraction did not show enzyme activity inhibition effects, while the ethylacetate and n-butanol fractions showed activity inhibitory effects.

The following tests were conducted in order to clarify the active ingredients of ethylacetate and n-butanol fractions showing enzyme activity inhibition effects. Silica gel column chromatography was conducted on the obtained ethylacetate fraction.

Silica chloroform, silica ethylacetate, silica acetone and silica methanol fractions were respectively obtained by sequentially eluting with chloroform, ethylacetate, acetone and methanol as an elution solvent. Although inhibitory effects were shown in silica ethylacetate (hereinafter referred to as silicaethylacetate) fractions as a result of examining inhibitory effects of these fractions on collagenases activities, they were not superior to *Ulmus* cortex primary extract and doxycycline (see the following Experiment

2).

The silica ehtylacetate fractions were analyzed using HPLC/ESI mass spectrometer, Finnigan LCQ, and thin layer chromatography to obtain the results of Fig. 1, which indicates the presence of procyanidin monomers having low molecular weights, two kinds of procyanidin dimmers, and unconfirmed materials.

The results of collagenase activity inhibition tests on each of the ingredient did not show good effects compared to those of doxycycline and *Ulmus* cortex primary extract which have conventionally been used as medicines, like the results of silica ethylacetate fractions.

On the other hand, Sephadex LH-20 column chromatography was conducted on n-butanol fractions obtained by solvent-fractionating a primary extract. The primary extract was sequentially eluted with 100 wt% methanol, fractionated and recombined through chromatography to designate them as fractions 1, 2, 3 and 4.

A 20% methanol fraction, a 50% methanol fraction, an 80% methanol fraction and a 100% methanol fraction were respectively obtained by eluting the primary extract with 20 wt% of methanol, 50 wt% of methanol, 80 wt% of methanol and 100 wt% of methanol as elution solvents, by different method.

As the results of testing collagenase activity inhibition on the obtained fractions 1, 2, 3, 4 and methanol fractions, fractions 1, 2, and 3, and 20 wt% and 50 wt% methanol fractions did not exibit any effect, while fraction 4, and 80 wt% and 100 wt% methanol fractions exhibited effects. Particularly, fraction 4 and 100 wt% methanol fraction exhibited excellent

effects, showing higher enzyme inhibiting activities than the conventional doxycycline and epigallocatechin gallate medicines.

The presence of procyanidin could be confirmed by observing that the color of the obtained fraction 4 and 100 wt% of methanol fraction changed into navy blue when a 10% $FeCl_3$ solution was added thereto. It is referred to as procyanidin oligomer hereinafter.

Experiment 1-2 (analysis of the sructure of fraction 4: matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer)

A mass of $[M + sodium]^+$ in which sodium was added to the molecular weight of a procyanidin oligomer was detected by a PE Biosystems Voyager System 4095 (Perkin Elmer) using cyano-4-hydroxycinnamic acid as a matrix.

From the fact that the results of MALDI-TOF mass spectrometer on fration 4 showed that mass increased by 288 from 889(m/z) ion peak as shown in Fig. 2, fraction 4 can be estimated to be a procyanidin oligomer in which catechin or epicatechin monomers were connected by single bonds. The ion peak of 889 m/z corresponded to a trimer in which a sodium mass of 23 was added to a molecular weight of 866. If calculating the rest of the peaks in the same method, it can be seen that the peaks correspond to various oligomers in which 3 to 12 of procyanidin monomers are polymerized. Furthermore, from the fact that an expected peak in dimmers, a peak of 601 m/z, was not detected, it can be seen that the trimers to dodecamers were distributed in fraction 4 having excellent effects.

These results are very similar to the distribution of procyanidins

contained in cocoa and chocolate (J. Agric. Food Chem., 1999, 47, 490-496, U.S. Patent No. 5,877,206). This mass analyzing method can be utilized in the calculation of a distribution degree of molecules and a degree of polymerization since it neither makes fractions of procyanidin oligomer molecules nor generates multiple charges (Phytochemistry 2000; 54: 173-181, and Rapid Comm. Mass Spectrometry 1997; 11: 31-36). The results of fraction 4 were analyzed by peak height ratios and molecular weights, and presented in the following Table 1. From Table 1, it can be seen that an average molecular weight was 1,518 and an average degree of polymerization was 5.3.

[Table 1]

Polymers	(Molecular weight + Na^+) m/z	Molecular weights	Peak height ratios (%)
Trimers	889	866	16.5
Tetramers	1177	1154	28.3
Pentamers	1465	1442	22.6
Hexamers	1754	1730	12.3
Heptamers	2042	2018	8.8
Octamers	2331	2306	6.2
Nonamers	2619	2594	2.1
Decamers	2908	2882	1.6
Undecamers	3193	3170	0.8
Dodecamers	3481	3458	0.8

Experiment 1- 3 (analysis of the structure of fraction 4: HPLC/ESI mass spectrometer, Finnigan LCQ)

In order to obtain structure information complementary to the analysis results by the MALDI-TOF mass spectrometer, HPLC/ESI

mass anaylsis which is used to measure the molecular weights of biosubstances which are not ionized well, such as procyanidin oligomers (Phytochemistry 1997; 44: 351-357, and J. Agric. Food Chem., 1999; 47: 3693-3710), and generates multiple charges was conducted.

An HP 1100 series HPLC (Hewlett-Packard, Palo Alto, U.S.A) was used as a chromatography. Silica (ZoRBOX Sil, 4.6 X 250 mm, 5 um) was usd as a column, and solvent conditions were set such that a ratio of solvent 1 (CH_2Cl_2 : CH_3OH : H_2O : CH_3COOH = 82 : 14 : 2 : 2) to solvent 2 (CH_3OH : H_2O : CH_3COOH = 96 : 2 : 2) changed from 100 : 0 at the beginnings to 12 : 88 at 50 minutes, and the flow rate thereof was 1 m/min. 280 nanometer absorption values of procyanidin oligomers were detected using a DAD detector. The results obtained by a mass spectrometer connected to the DAD detector were compared with those obtained by a MALDI-TOF mass spectrometer. In a selective mass tracing mode, $[M - H]^-$ mass in which hydrogen was excluded from a procyanidin oligomer molecular weight, $[M + TFA^-]^-$ mass in which one molecule of trifluoroacetic acid (TFA) was added to a procyanidin oligomer molecular weight, and mass peaks of $[M - H + TFA^-]^{-2}$ and $[M + 2\ TFA]^{-2}$ in which charge numbers are expected to be 2 were used. For example, pentamers were traced by designating a $[M - H]^-$ mass of 1441 and a $[M + TFA^-]^-$ mass of 1555, and trimers to hexamers were traced by designating their masses in the same method, as shown in Fig. 3. Since heptamers to dodecamers have charge number of 2 instead of 1 (J. Agric. Food Chem., 1999, 47, 490-496), corresponding masses, for example a 1065 peak of $[M - H + TFA^-]^{-2}$ and a 1122 peak of $[M + 2\ TFA]^{-2}$ in

heptamers, were traced, and octamers to dodecamers were traced by the same method.

As shown in Fig. 4, expected peaks of procyanidin oligomers of trimers to dodecamers were detected in an active fraction 4, and it could be seen that the distribution of ions having expected masses shifted from the initial part to the latter part of the chromatograph of fraction 4 as the degree of polymerization increased from 3.

The following Table 2 shows the ion masses of expected procyanidin oligomer in fraction 4 and ion types detected by an electron spray ionization mass spectromer, and blanks mean that they were not detected

[Table 2]

Polymers	$(M-H)^-$	$[M + TFA]^-$	$[M-H + TFA]^{-2}$	$[M + 2TFA]^{-2}$	Molecular weights
Trimers	865	979			866
Tetramers	1153	1267			1154
Pentamers	1441	1555		834	1442
Hexamers	1729	1843	921	978	1730
Heptamers			1065	1122	2018
Octamers			1209	1266	2306
Nonamers			1353	1410	2594
Decamers			1497	1554	2882
Undecamers			1641	1698	3170
Dodecamers			1785	1842	3458

Fig. 5 shows the results of detecting mass peaks in a scan mode at a retention time zone on a chromatogram near designated mass peaks using HPLC/ESI mass spectrometer, indicating that the main ingredient of fraction 4 is procyanidin oligomer since the main mass peaks are 1153 ($[M-H]^-$)

and 1267 ([M + TFA^-] $^-$) in case the retention time range was set for tetramers, and 1441 ([M – H] $^-$) and 1555 ([M + TFA^-] $^-$) in case the retention time range was set for pentamers. Thus, it can be seen that the main ingredient of fraction 4 is procyanidin oligomer.

Considering the results of a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer and a high performance liquid chromatography/electron spray ionization (HPLC/ESI) mass spectrometer, the ingredient of fraction 4 having excellent MMP inhinitory effects is procyanidin oligomer mixture in which 3 to 12 of flavan-3-ol monomers are connected through single bonds, having a molecular weight of 1,518 and average polymerization degree of 5.3.

Experiment 1-4

(Analysis of the monomers constituting procyanidin oligomer)

A phloroglucinol acidolysis was carried out in order to analyze monomers constituting procyanidin oligomer of fraction 4 (J. Chromatogr., 1992; 594, 117-123).

Phloroglucinol acidolysis is a reaction in which phloroglucinol disconnects bonds between monomers in polymers to cause an addition reaction when a polymer and a phloroglucinol are reacted under acidic conditions, thereby producing monomer-phloroglucinol compound as a reaction product. As a result of separating and comparing the reaction products produced after reacting phloroglucinol and fraction 4 under acidic conditions using HPLC, phloroglucinol derivatives of epicatechin, catechin gallate, epicatechin gallate, gallocatechin gallate or epigallocatechin gallate

were not detected beside peaks estimated to be of catechin-phloroglucinol. The purified estimated catechin-phloroglucinol was analyzed using ESI mass spectrometer to identify the molecular weight of 414 from an expected mass peaks.

Carbon and hydrogen nuclear magnetic resonance methods (^{13}C, ^{1}H NMR) were used to distinguish catechin from epicatechin. From the fact that a chemical shift of carbon No. 2 was different between catechin and epicatechin depending on chemical shift, i.e., alignment of phloroglucinol connected with carbon No. 4 within a heterocyclic ring (J. Chem. Soc. Perkin, Trans., I 1980; 2278-2286, J. Chem. Soc. Perkin, Trans., I 1983; 1535-1543, and J.C.S. Perkin I 1982, 1217-1221), it could be seen that chemical shift of carbon No. 2 of phloroglucinol derivative is 83.4 ppm corresponding to catechin-4-phloroglucinol.

Therefore, it could be seen that the first monomer of procyanidin oligomers of the present invention is a catechin.

Considering the results of the MALDI-TOF and HPLC/ESI mass spectrometers and NMR spectrometry, ingredients of fraction 4 having excellent MMP inhibitory effects are procyanidin oligomers in which 3 to 12 of flavan-3-ol monomers are connected through single bonds, having an average molecular weight of 1,518 and an average polymerization defree of 5.3, differently from those of silica ethylacetate having low effects.

Experiment 2

(Evaluation of inhibitory effects of procyanidin oligomer fractions on MMP activities using a red collagen matrix of Azocoll (Anal. Biochem., 1984, 136;

446-450)).

Inhibitory effects of procyanidin oligomer fractions on collagenolytic proteinase (from Kamchatka crabs, bought from Sigma Corporation), MMP-1 (fibroblast collagenase), and MMP-8 (polymorphonuclear leukocyte collagenase) were measured by the following method.

100 ㎕ of Azocoll solution that is a 2% red collagen matrix was added to each 1.5 ml Eppendorf tube. One Eppendorf tube was used as a blank. And collagenolytic enzyme standard solutions bought from Sigma were respectively added to three tubes such that each tube contained 10, 100 and 200 ppm of the solution, in order to draw up enzyme standard activity curves. 100 ㎕ of each collagenolytic enzyme standard solution was added to other tubes, and *Ulmus* cortex primary extract, silica ethylacetate fraction, procyanidin oligomer fraction, tetracycline, minocycline and doxycycline, and epigallocatechin gallate were added thereto such that their concentrations corresponded to 0.0001, 0.001, 0.005, and 0.01 weight% respectively.

A buffer solution (0.05 M Tris-HCl, 1 nM $CaCl_2$, pH 7.8) was added to each tube such that the total reactant solution amounted to 500 ㎕, and they were reacted in an incubator at a temperature of 37 ℃ for 18 hours, and then Eppendorf tubes were centrifuged at 10,000 g for 5 minutes to precipitate collagens which were not decomposed. And then, supernatant containing decomposed collagen was taken and the absorbance thereof was measured at 540 nm to draw up standard activity curves, and enzyme activities were converted from the drawn standard curves to compare and

estimate the enzyme activities of the test groups and control. Activity inhibition effect tests on MMP-1 and MMP-8 were performed by the same method as mentioned above. Each enzyme standard solution was bought from Calbiochem The test results are as follows.

The results of inhibitory effects on the collagenolytic activities by various agents are presented in the following Table 2, wherein the enzyme inhibition rates were calculated according to the following Equation 1:

[Equation 1]

Enzyme inhibition rate (%) = 100 – (enzyme activities of test groups X100 ÷ enzyme activities of control)

[Table 2]

test groups (enzyme inhibition rate, %)	0.0001%	0.001%	0.005%	0.01%
Tetracycline	0	0	0	32
Minocycline	0	0	24	43
Doxycycline	0	0	54	66
Epigallocatechin gallate	0	29	60	80
Procyanidin oligomer fractions	20	63	86	100
Silica ethylacetate fractions	0	0	49	45
Ulmus cortex primary extracts	0	10	68	68

Furthermore, the results of evaluating inhibitory effects of procyanidin oligomer fractions on MMP-1 (fibroblast collagenase) activities are presented in the following Table 3, wherein the enzyme inhibition rates were calculated

according to the Equation 1.

[Table 3]

test groups (enzyme inhibition rate, %)	0.0001%	0.001%	0.005%	0.01%
Tetracycline	0	0	0	24
Minocycline	0	0	25	55
Doxycycline	0	0	47	83
Epigallocatechin gallate	10	10	69	90
Procyanidin oligomer fractions	25	68	86	100
Silica ethylacetate fractions	0	0	37	70
Ulmus cortex primary extracts	0	0	50	90

Furthermore, the results of evaluating inhibitory effects of procyanidin oligomer fractions on MMP-8 (polymorphonuclear leukocyte collagenase) activities are presented in the following Table 4, wherein enzyme inhibition rates were calculated according to the Equation 1.

[Table 4]

test groups (enzyme inhibition rate, %)	0.0001%	0.001%	0.005%	0.01%
Tetracycline	0	0	10	70
Minocycline	0	0	23	50
Doxycycline	0	0	32	81
Epigallocatechin gallate	22	49	80	100
Procyanidin oligomer fractions	45	80	89	100
Silica ethylacetate fractions	0	0	27	53

Ulmus cortex primary extracts	0	30	69	95

Considering all the test results, procyanidin oligomer fractions showed higher inhibitory effects on the activities of collagenolytic enzyme, MMP-1 and MMP-8 than epigallocatechin gallate, a green tea ingredient and doxycycline which are already known, they also showed higher effects than *Ulmus* cortex primary extract and silicaethylacetate. Accordingly, it is judged that procyanidin oligomer fraction is a main active ingredient for MMP emzyme inhibition of *Ulmus* cortex.

Experiment 3

(Evaluation of inhibitory effects of procyanidin oligomer fractions on MMP activities using a collageno kit (CLN-100, Japan Cosmobio)

This test method is based on the fact that only decomposed collagens are degenerated at 35 ℃ and extracted by ethanol when collagen type-I that is conjugated with fluorescent substance fluorosceiniso thiocyanate (FITC) is treated with collagenase. In this test, the reactant solution was centrifuged, and then fluorescence intensity (FI) of supernatant was measured at 520 nm (EM) / 495 (EX) by a fluorescence spectrometer (Inflammation, 1994, 18; 613-623). 200 ㎕ of 0.05% FITC-collagen were respectively added to each 1.5 ㎖ Eppendorf tube. One Eppendorf tube was used as a blank, and a MMP-1 standard enzyme solutions bought from Calbiochem were respectively added to three tubes such that the solutions amounted to 10,. 100 and 200 ppm, respectively, in order to draw up enzyme

standard activity curves. 100 ㎕ of enzyme standard solution were respectively added to other tubes, and procyanidin oligomer fraction, silica ethylacetate fraction, tetracycline, minocycline and doxycycline were added thereto such that they amounted to 0.001, 0.01, 0.03, and 0.05 wt%, respectively. A buffer solution (0.05 M Tris-HCl, 1 mM $CaCl_2$, pH 7.8) was added to each tube such that each reactant solution amounted to 500 ㎕, and they were reacted in an incubator at a temperature of 37 ℃ for 18 hours. 10 ㎕ of 80 mM o -phenanthroline dissolved in 50 wt% ethanol were added as a reaction stopping solution, it was maintained in an incubator at a temperature of 37 ℃ for 18 hours, and cooled. 0.5 ml of 70 wt% ethanol were respectively added to the cooled Eppendorf tubes, and they were centrifuged at 3,000 rpm for 10 minutes. After centrifuging, 500 ㎕ of supernatant were taken from each tube and fluorescence intensity (FI) was measured at 520 nm (EM) / 495 (EX) with a fluorescence spectrometer to draw up standard curves relating to enzyme activities. Enzyme activity concentrations were converted from the standard curve to compare enzyme activities of the test groups and the control. Activity inhibition tests on MMP-8 were conducted by the same method as mentioned above. The test results are as follows.

The evaluation of inhibitory effects of procyanidin oligomer fractions on MMP-1 activities are presented in the following Table 5, wherein the enzyme inhibition rates were calculated according to the Equation 1.

[Table 5]

test groups (enzyme inhibition rate, %)	0.001%	0.01%	0.03%	0.05%
Tetracycline	0	23	44	65
Minocycline	0	36	55	79
Doxycycline	0	69	68	95
Procyanidin oligomer fractions	45	89	100	100
Silica ethylacetate fractions	0	38	35	59

Furthermore, the evaluation of inhibitory effects of procyanidin oligomer fractions on MMP-8 activities are presented in the following Table 6, wherein enzyme inhibition rates were calculated according to the Equation 1.

[Table 6]

test groups (enzyme inhibition rate, %)	0.001%	0.01%	0.03%	0.05%
Tetracycline	0	20	55	64
Minocycline	0	40	68	85
Doxycycline	0	65	64	90
Procyanidin oligomer fractions	63	92	95	100
Silica ethylacetate fractions	0	56	50	63

According to the above test results, procyanidin oligomer fractions also showed higher inhibitory effects on MMP activities than tetracyclines, and they showed almost 100% activity inhibitory effects on two enzymes, i.e., MMP-1 and MMP-8 at a concentration of 0.03 weight%. It can be seen that the inhibitory effects of silica ethylacetate fractions consisting of flavan-3-ol monomers, two kinds of dimers, some trimers and unidentified materials are

remarkably less than those of procyanidin oligomers of the present invention. Out of the tetracyclines, doxycycline showed highest effects, showing 90% activity inhibitory effects both on MMP-1 and MMP-8 at a concentration of 0.05 wt%, and tetracycline and minocycline showed lower enzyme activity inhibitory effects.

Experiment 4

(The evaluation of Inhibitory effects of procyanidin oligomer fractions on the activities of collagenases type IV (MMP-2 and MMP-9) using Zymography)

MMP-2 and MMP-9 enzyme standard solutions bought from Calbiochem were mixed with a buffer solution (2.5% SDS(sodium dodecylsulfate), 50 mM Tris-HCl, pH 6.8, 10% glycerol, 0.005% bromophenol blue, 3% sucrose), and then electrophoresis was conducted on a gel plate containing 0.2% gelatin that is collagenase type-IV (8% SDS-polyacrylamide gel). After electrophoresis, the gel was washed with 50 mM Tris-HCl (pH 7.5) buffer solution containing 2.5% Tritox X-100 twice for 30 minutes to remove SDS. The gel was cut to several pieces lengthwise, and they were then put into a reaction buffer solution comprising an enzyme standard solution and various concentrations of 0.0001, 0.001, 0.005 and 0.01 % of procyanidin oligomer fractions, tetracycline, minocycline, and doxycycline, and they were reacted at a temperature of 37 ℃ for 18 hours.

Then, the gel was dyed with 0.1% Coomasie brilliant blue and decolored to measure gelatin decomposing performance by densitometer, thereby drawing up standard activity curves. And then, enzyme activities of the test groups and the control were compared by calculating them from the

standard curves. Activity inhibition tests on collagenase type IV, i.e., MMP-9 were conducted by the same method as described in the above. The results are as follows.

The evaluation of inhibitory effects of procyanidin oligomer fractions on MMP-2 activities are presented in the following Table 7, wherein enzyme inhibition rates were calculated according to the Equation 1.

[Table 7]

test groups (enzyme inhibition rate, %)	0.0001%	0.001%	0.005%	0.01%
Tetracycline	0	0	19	45
Minocycline	0	0	26	46
Doxycycline	0	12	45	82
Procyanidin oligomer fractions	21	59	87	100
Silica ethylacetate fractions	0	0	30	36

Furthermore, the evaluation of inhibitory effects of procyanidin oligomer fractions on MMP-9 activities are presented in the following Table 8, wherein enzyme inhibition rates were calculated according to the Equation 1.

[Table 8]

test groups (enzyme inhibition rate, %)	0.0001%	0.001%	0.005%	0.01%
Tetracycline	0	0	15	68
Minocycline	0	0	30	43
Doxycycline	0	0	45	89
Procyanidin oligomer fractions	30	45	89	100
Silica ethylacetate fractions	0	0	43	56

Considering all the test results, procyanidin oligomer fractions showed higher inhibitory effects on enzyme activities of collagenases type IV such as MMP-2 and MMP-9 than tetracyclines, and they showed 100% inhibitory effects both on activities of MMP-2 and MMP-9 at a concentration of 0.01%. Out of tetracyclines, doxycycline showed highest effects, and minocycline and tetracycline showed lower effects. From the fact that the inhibitory effects of silica ethylacetate fractions consisting of flavan-3-ol monomer, two kinds of dimmers, some trimers and unidentified material were remarkably lower than those of procyanidin oligomer of the present invention, it could be seen that the *Ulmus* primary extract titer was mainly contributed by procyanidin oligomers of the present invention.

Experiment 5

(Peridontal ligament cell secreted MMP inhibition test)

Periodontal ligament tissues spotted on the extracted tooth surface were scraped with a scalpel and cultured in a medium. Cells of 6^{th} to 8^{th} generations grown from tissues to outside were used.

The test was conducted using periodontal ligament tissue supernatant as a zymogen of Experiment 3. MMP secreted from periodontal ligament seems to be collagenase type IV, considering its molecular weight, and the fact that its titer is fully inhibited by EDTA and it is not inhibited by other proteinase inhibitor such as PMSF (serine based proteinase inhibitor) and pepstatin

As shown in Fig. 6, the effects of procynidin oligomer were 10 times

superior to those of doxycycline.

Experiment 6

(Effects on paradentitis bacteria collagenases)

In order to test inhibition of collagenase activity from two kinds of periopathogenic bacteria (Porphyromonas gingivalis Treponema denticola) cultured extracts, radioactive material labelled collagen matrixes (^{3}H-collagen collagen type 4(N-propionate-2,3-^{3}H-propionated, mCi/ml: NEN Life Science Products, Boston, MA) were mixed with various concentrations of medicines and incubated for 18 hours. Collagens that were not decompesed were precipitated with 0.05% tannic acid and trichloroacetic acid, and then radioactivities remained in a supernatant was measured to compare inhibitory activities of medicines.

It was also found that procyanidin oligomers of the present invention were 10 times or more superior to silica ethylacetate fractions and doxycycline.

Experiment 7

(Selectivity of procyanidin oligomers on enzyme inhibition)

In order to know whether or not procyanidin is a nonspecific enzyme inhibitor due to the general protein binding, the collagenase inhibitory effects of procyanidin using Azocoll as in Experiment 2 and elastase inhibitory effects thereof were compared with those of doxycycline.

Elastin to which congo red is bound (elastin-congo red, Sigma) was mixed with reactants solutions prepared with various medicine concentrations (10 Mm TES buffer, pH 7.0). Elastase (Sigma E-150) was added to the

mixture and was cultured for 18 hours. Then, elastins which were not decomposed were precipitated and the absorbance of the supernatant thereof was measured at 495 nm. Relative reaction inhibition degrees are presented in the following Table 9 by % inhibitory activity, taking the enzyme activities of the control in which only enzymes exist and medicines do not exist, namely decomposition was sufficiently progressed as 100 i.e., 0% inhibition.

Enzyme inhibiton rates were calculated according to the Equation 1.

[Table 9]

Classification (enzyme inhibition rates, %)		Control	0.001%	0.003%	0.007%	0.01%	0.03%	0.07%	0.1%
Collagenolytic enzyme	Doxycycline	0	0	24	64	60	85	93	100
	Procyanidin oligomer	0	63	86	89	100	100	100	100
Elastase	Doxycycline	0	10	40	100	100	100	100	100
	Procyanidin oligomer	0	0	0	10	20	40	60	100

From the above results, it can be seen that procyanidin is not a nonspecific enzyme inhibitor due to the general protein bonds, since the inhibitory effects of procyanidin oligomers on collagenolytic enzymes are superior to those of doxycycline, but the inhibitory effects thereof on elastase are lower than those of doxycycline.

[Examples]

In the following Examples, the effects of ointments of Examples 1 to 8 of the present invention on collagenolytic enzyme activities were evaluated.

Procyanidin oligomer fractions of the present invention can be

prepared in any form for inhibiting activities of MMP such as collagenase. These forms possibly include tablets, capsules, powders, ointments, solutions, gels, pastes, patches, granules, etc. However, ointments were tested in the present invention.

The contents of procyanidin oligomer fractions are 0.0001 to 5 wt%, preferably 0.01 to 1 wt%. When the contents thereof is less than 0.0001 wt%, enzyme activity inhibitory effects or efficacies cannot be expected, and when the contents exceed 5 wt%, the color of procyanidin oligomer fractions excessively changed into brown, makng it difficult to use them.

The ointment compositions used in the following Examples and Comparatice Examples were prepared by the following methods.

An ointment composition was prepared by dissolving a mixture comprising a procyanidin oligomer fraction, pluronic, lower alcohol, glycerin, gelatin, pectin, carboxymethylcellulose, poloxamer (Poloxamer 407), monoglyceride (Myverol 18-99), polypropylene glycol or polyethylene glycol, menthol and a preservative in a buffer solution and gellating the dissolved mixture. In Comparative Examples, the ointment compositions were prepared by the same method as above, except that doxycyclines were used instead of procyanidin oligomer fractions.

0 to 20 wt% of ethanol, isopropyl alcohol or a mixture thereof can be used as the lower alcohol, and 5 to 30 wt% of pluronic F-127 or pluronic F-108 can be used as pluronic derivatives which are used to enhance stability of an ointment composition. A wetting agent is used to maintain the conditions of composition and to prevent it from drying, and it is selected from

the group consisting of glycerine, sorbitol, polyethylene glycol, propylene glycol, poloxamer (Poloxamer 407), monoglyceride (Myverol 18-99), and a mixture thereof, and is used in an amount of 5 to 30 wt%. Particularly, polyethylene glycol includes polyethylene glycol 200, polyethylene glycol 400, polyethylene glycol 600, or polyethylene glycol 1000.

In addition, 1 to 20 wt% of gelatin or pectin, or a mixture thereof are used as a system for deliverying medicinal efficacies of active ingredient into skin tissues. A buffer agent adjusting the pH of the ointment compositions includes alkaline metal salts of o -phosphates, particularly primary sodium phosphate, secondary sodium phosphate, tertiary sodium phosphate, citric acid and sodium citrate, phosphoric acid, hydrochloric acid, sodium hydroxide or sodium pyrophosphate, pyrophosphate, etc. In ointment compositions of the Examples, two kinds of primary sodium phosphate, secondary sodium phosphate, and tertiary sodium phosphate were properly mixed and the pH of the mixture was adjusted to 5 to 8.0 before using.

Additionally, sodium metabisulfite is used to prevent discoloration of procyanidin oligomer fractions, and it is used in an amount of 0.05 to 1 wt% of the ointment composition. Natural flavors, peppermint and spearmint oil, are commonly used as a flavor, and it is used in an amount of 0.1 to 1 wt% of the ointment composition. Furthermore, in order to prevent contaminations by microorganisms possibly occurred during the preparation and uses of oinment compositions, methyl paraoxybenzoate, propyl paraoxybenzoate, benzoic acid, sodium benzoate, salicylic acid, which are permitted to be generally used in food and medicines, or a mixture thereof is used in an

amount of 0.01 to 0.5 wt%.

Examples 1 to 8 and Comparative Examples 1to 8

Ointment compositions of each Example and Comparative Example are presented in the following Tables 10 to 13.

[Table 10]

Classifications (wt%)	Constituents	EXAMPLE 1	EXAMPLE 2	COMPARATIVE EXAMPLE 1	COMPARATIVE EXAMPLE 2
Wetting agent	Glycerin	5	-	5	-
	Polyethylene glycol	-	5	-	5
	Carboxymethylcellulose	-	-	-	-
	Poloxamer 407	0.1	-	0.1	-
	Monoglyceride (Myverol 18-99)	10	15	10	15
Medicinal efficacy delivery system	Gelatin	5	5	5	5
	Pectin	5	5	5	5
Surfactant	Pluronic F-127	20	-	20	-
	Pluronic F-108	-	20	-	20
Lower alcohol	Ethanol	5	-	5	-
	Isopropyl alcohol	-	5	-	5
Preservative	Methyl paraoxybenzoate	0.1	0.1	0.1	0.1
	Propyl paraoxybenzoate	0.05	0.05	0.05	0.05
Antidiscoloring agent	Sodium metabisulfite	0.1	0.2	0.1	0.2
Buffer solution	Primary sodium phosphate	0.1	0.2	0.1	0.2
	Tertiary sodium phosphate	0.05	0.05	0.05	0.05
Medicinal composition	Procyanidin oligomer fraction	0.0001	0.001	-	-
	Doxycycline	-	-	0.0001	0.001
Flavor	-	0.75	0.75	0.75	0.75
Edible pigment	-	0.0002	-	0.0002	-

Purified water	-	Balance	Balance	Balance	Balance

[Table 11]

Classifications (wt%)	Constituents	EXAMPLE 3	EXAMPLE 4	COMPARATIVE EXAMPLE 3	COMPARATIVE EXAMPLE 4
Wetting agent	Glycerin	10	-	10	-
	Polyethylene glycol	-	5	-	5
	Carboxymethylcellulose	0.5	0.1	0.5	0.1
	Poloxamer 407	-	-	-	-
	Monoglyceride (Myverol 18-99)	10	20	10	20
Medicinal Efficacy delivery system	Gelatin	3	2	3	2
	Pectin	3	2	3	2
Surfactant	Pluronic F-127	15	-	15	-
	Pluronic F-108	-	15	-	15
Lower alcohol	Ethanol	5	-	5	-
	Isopropyl alcohol	-	5	-	5
Preservative	Methyl paraoxybenzoate	0.1	0.1	0.1	0.1
	Propyl paraoxybenzoate	0.05	0.05	0.05	0.05
Antidiscoloring agent	Sodium metabisulfite	0.1	0.2	0.1	0.2
Buffer solution	Primary sodium phosphate	0.1	0.2	0.1	0.2
	Tertiary sodium phosphate	0.05	0.05	0.05	0.05
Medicinal composition	Procyanidin oligomer fraction	0.005	0.01	-	-
	Doxycycline	-	-	0.005	0.01
Flavor	-	0.75	0.75	0.75	0.75
Edible pigment	-	0.0002	-	0.0002	-
Purified water	-	Balance	Balance	Balance	Balance

[Table 12]

Classifications (wt%)	Constituents	EXAMPLE 5	EXAMPLE 6	COMPARATIVE EXAMPLE 5	COMPARATIVE EXAMPLE 6
Wetting agent	Glycerin	15	-	15	-
	Polyethylene glycol	-	5	-	5
	Carboxymethylcellulose	-	-	-	-
	Poloxamer 407	0.5	1	0.5	1
	Monoglyceride (Myverol 18-99)	5	5	5	5
Medicinal Efficacy delivery system	Gelatin	1	10	1	10
	Pectin	1	5	1	5
Surfactant	Pluronic F-127	10	-	10	-
	Pluronic F-108	-	10	-	10
Lower alcohol	Ethanol	5	-	5	-
	Isopropyl alcohol	-	5	-	5
Preservative	Methyl paraoxybenzoate	0.1	0.1	0.1	0.1
	Propyl paraoxybenzoate	0.05	0.05	0.05	0.05
Antidiscoloring agent	Sodium metabisulfite	0.1	0.2	0.1	0.2
Buffer solution	Primary sodium phosphate	0.1	0.2	0.1	0.2
	Tertiary sodium phosphate	0.05	0.05	0.05	0.05
Medicinal composition	Procyanidin oligomer fraction	0.05	0.1	-	-
	Doxycycline	-	-	0.05	0.1
Flavor	-	0.75	0.75	0.75	0.75
Edible pigment	-	0.0002	-	0.0002	-
Purified water	-	Balance	Balance	Balance	Balance

[Table 13]

Classifications (wt%)	Constituents	EXAMPLE 7	EXAMPLE 8	COMPARATIVE EXAMPLE 7	COMPARATIVE EXAMPLE 8

Wetting agent	Glycerin	20	-	20	-
	Polyethylene glycol	-	5	-	5
	Carboxymethylcellulose	-	-	-	-
	Poloxamer 407	-	-	-	-
	Monoglyceride (Myverol 18-99)	10	20	10	20
Medicinal Efficacy delivery system	Gelatin	5	20	5	20
	Pectin	5	5	5	5
Surfactant	Pluronic F-127	5	-	5	-
	Pluronic F-108	-	5	-	5
Lower alcohol	Ethanol	10	-	10	-
	Isopropyl alcohol	-	10	-	10
Preservative	Methyl paraoxybenzoate	0.1	0.1	0.1	0.1
	Propyl paraoxybenzoate	0.05	0.05	0.05	0.05
Antidiscoloring agent	Sodium metabisulfite	0.1	0.2	0.1	0.2
Buffer solution	Primary sodium phosphate	0.1	0.2	0.1	0.2
	Tertiary sodium phosphate	0.05	0.05	0.05	0.05
Medicinal composition	Procyanidin oligomer fraction	1	5	-	-
	Doxycycline	-	-	1	5
Flavor	-	0.75	0.75	0.75	0.75
Edible pigment	-	0.0002	-	0.0002	-
Purified water	-	Balance	Balance	Balance	Balance

The procedures and results of testing inhibitory effects of each oinment on collagenolytic enzymes were as follow.

100 ㎕ of 2% Azocoll solution that is a red collagen matrix were respectively added to 20 of 1.5 ml Eppendorf tubes. One Eppendorf tube was used as a blank, and a collagenolytic enzyme standard solution bought

from Sigma was added to each of three tubes such that they amounted to 10, 100, and 200 ppm, in order to draw a standard activity curve of the enzyme. Each of the test group ointments from Examples 1 to 8 and control ointments from Comparative Examples 1 to 8 was mixd with distilled water in a ratio of 1:2 and homogenized, and 10 ㎕ of supernatant obtained by centrifuging at 5,000 g for 10 minutes and 100 ㎕ enzyme standard solution were added to each of the 16 remaining tubes. A buffer solution (0.05 M Tris-HCl, 1 mM $CaCl_2$, pH 7.8) was added to each tube such that the total reactant solution amounted to 500 ㎕ and reacted in an incubator at a 37 ℃ temperature for 18 hours, and then Eppendorf tubes were centrifuged at 10,000 g for 5 minutes to precipitate collgens which were not decompose. Supernatants containing decomposed collagens were taken and the absorbance was measured at 540 nm to draw up a standard activity curve, and enzyme activities of the test groups and the controls were compared and evaluated by converting enzyme activity concentrations from the standard curve. Thereby the following results were obtained, wherein the enzyme inhibition rates were calculated according to the Equation 1.

[Table 14]

Classification	Enzyme inhibition rate (%)
EXAMPLE 1	0
EXAMPLE 2	0
EXAMPLE 3	48
EXAMPLE 4	80
EXAMPLE 5	100
EXAMPLE 6	100
EXAMPLE 7	100

EXAMPLE 8	100
COMPARATIVE EXAMPLE 1	0
COMPARATIVE EXAMPLE 2	0
COMPARATIVE EXAMPLE 3	5
COMPARATIVE EXAMPLE 4	43
COMPARATIVE EXAMPLE 5	80
COMPARATIVE EXAMPLE 6	100
COMPARATIVE EXAMPLE 7	100
COMPARATIVE EXAMPLE 8	100

As shown in the above test results, the compositions containing procyanidin oligomer fractions of Examples according to the present invention showed higher inhibitory effects on collagenolytic enzyme activities than those containing doxycycline of Comparative Examples at a procyanidin concentration of 0.05 % or below.

Accordingly, a medicine containing procyanidin oligomers of the present invention as an active ingredient can be used as a medicine for preventing and treating diseases related to MMP activities such as metastasis of cancer, rheumatoid arthritis, diabetes, inflammation, corneal ulcer, epidermal ulcer, and gastric ulcer, skin wrinkling and aging, paradentitis, osteoporosis, acne, trauma and burn healing, hyperparathyroidism, etc.

While the present invention has been described in detail with reference to the preferred embodiments, those skilled in the art will appreciate that various modifications and substitutions can be made thereto without departing from the spirit and scope of the present invention as set forth in the appended claims.

WHAT IS CLAIMED IS:

1. A procyanidin oligomer which inhibits activities of matrix metalloproteinase (MMP).

2. The procyanidin oligomer according to claim 1, wherein the matrix metalloproteinases is selected from the group consisting of collagenolytic proteinase, MMP-1, MMP-8, type IV collagenase of MMP-2 and MMP-9, and bacteria secreted collagenase.

3. The procyanidin oligomer according to claim 1, wherein the procyanidin oligomer is an n-butanol fraction which is prepared by solvent-fractionating a primary extract which is extracted from *Ulmus* cortex that is the roots or barks of plants of genus *Ulmus* using a polar solvent.

4. The procyanidin oligomer according to claim 1, wherein the procyanidin oligomer is a preparation which is concentrated by sequentially eluting n-butanol fraction in methanol increasing order and recombining elutes based on thin layer chromatography when an n-butanol fraction which is prepared by solvent- partitioning an extract which is extracted from *Ulmus* cortex that is the roots or barks of plants of genus *Ulmus* using a polar solvent is further chromatographed on Sephadex LH-20 column with water-methanol mixed solution.

5. The procyanidin oligomer according to claim 1, wherein the procyanidin oligomer is a preparation which is concentrated by sequentially eluting the n-butanol fraction in 100% methanol and recombining elutes based on thin layer chromatography when the n-butanol fraction which is

prepared by solvent- partitioning an extract which is extracted from *Ulmus* cortex that is the roots or barks of plants of genus *Ulmus* using a polar solvent is further chromatographed on Sephadex LH-20 column with 100% methanol.

6. The procyanidin oligomer according to claim 1, wherein the procyanidin oligomer is an oligomer in which 3 to 12 monomers having flavan-3-ol as a backbone are connected through single bonds.

7. A pharmaceutical composition for preventing and/or treating disease caused by activities of matrix metalloproteinases (MMP), comprising a procyanidin oligomer as an active ingredient.

8. The pharmaceutical according to claim 7, wherein the disease is selected from the group consisting of metastasis of cancer, rheumatoid arthritis, diabetes, inflammation, corneal ulcer, epidermal ulcer, gastric ulcer, skin wrinkling and aging, paradentitis, osteoporosis, acne, wounds and burns, and hyperparathyroidism.

9. The pharmaceutical composition according to claim 7, wherein the matrix metalloproteinase is selected from the group consisting of collagenolytic proteinase, MMP-1, MMP-8, type IV collagenase of MMP-2 and MMP-9, and bacteria secreted collagenase.

10. The pharmaceutical composition according to claim 7, wherein the pharmaceutical composition is in the form of a tablet, capsule, powder, ointment, solution, gel, paste, patch, or granule.

11. The pharmaceutical composition according to claim 7, wherein the contents of the procyanidin oligomer is 0.0001 to 5 wt% of the

composition.

12. The pharmaceutical composition according to claim 7, wherein the procyanidin oligomer is an n-butanol fraction which is prepared by solvent- fractionating extracts which are extracted from *Ulmus* cortex that is the roots or barks of plants of genus *Ulmus* using a polar solvent

13. The pharmaceutical composition according to claim 7, wherein the procyanidin oligomer is a preparation which is concentrated by sequentially eluting the n-butanol fraction in methanol increasing order and recombining elutes based on thin layer chromatography when the n-butanol fraction which is prepared by solvent- partitioning an extract which is extracted from *Ulmus* cortex that is the roots or barks of plants of genus *Ulmus* using a polar solvent is further chromatographed on Sephadex LH-20 column with water-methanol mixed solution.

14. The pharmaceutical composition according to claim 7, wherein the procyanidin oligomer is a preparation which is concentrated by sequentially eluting the n-b utanol fraction in 100% methanol and recombining elutes based on thin layer chromatography when the n-butanol fraction which is prepared by solvent- partitioning an extract which is extracted from *Ulmus* cortex that is the roots or barks of plants of genus *Ulmus* using a polar solvent is further chromatographed on Sephadex LH-20 column with 100% methanol.

15. The pharmaceutical composition according to claim 7, wherein the procyanidin oligomer is an oligomer in which 3 to 12 monomers having flavan-3-ol as a backbone are connected through single bonds.

Fig 1

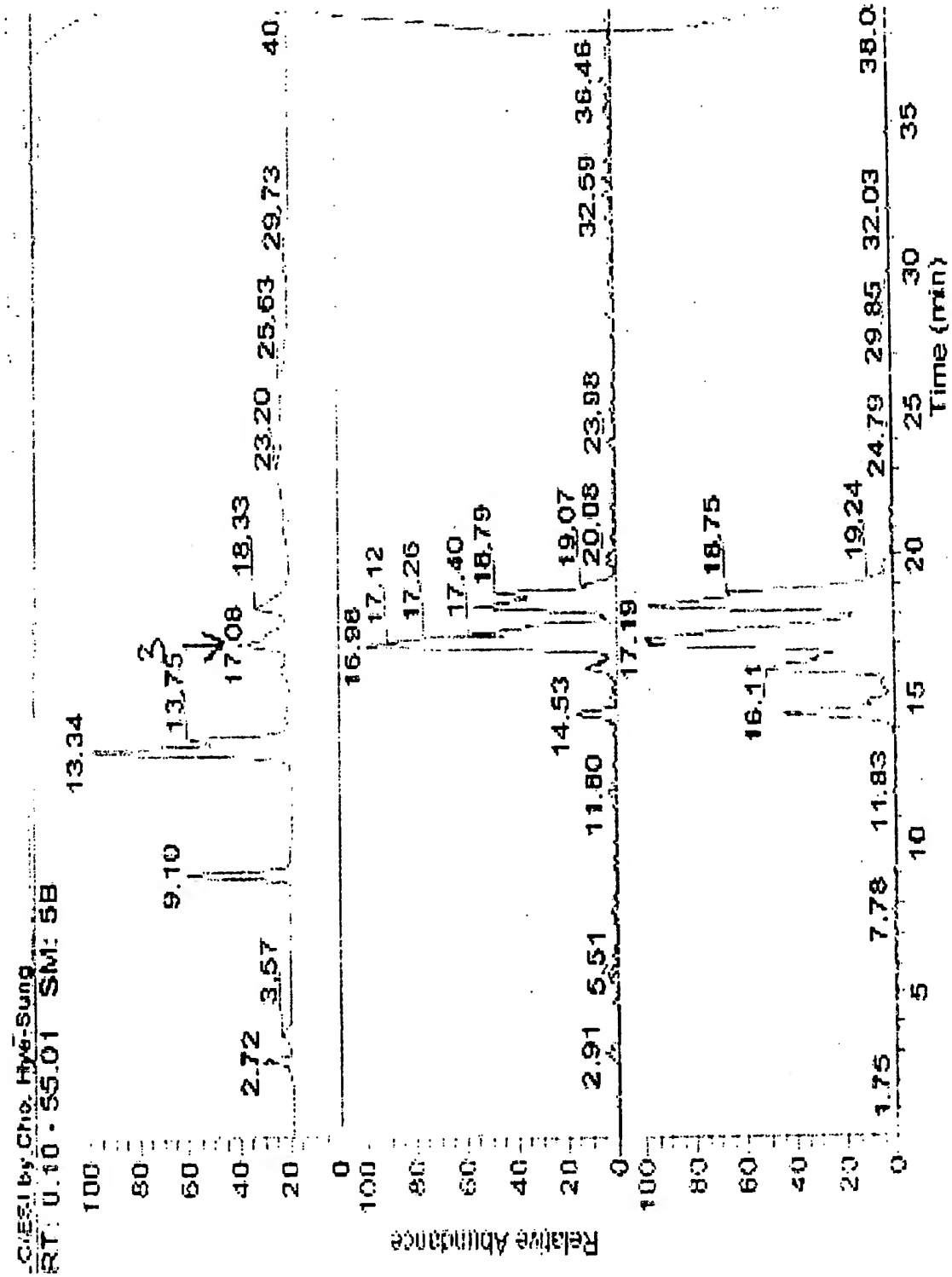

Relative Abundance
Time (min)
RT: 0.10 - 55.01 SM: 5B
CIESI by Cho, Hye-Sung
1.75
2.72
2.91
3.57
5.51
7.78
9.10
11.80
11.83
13.34
13.75
14.53
16.11
16.98
17.08
17.12
17.19
17.26
17.40
18.33
18.75
18.79
19.07
19.24
20.08
23.20
23.98
24.79
25.63
29.73
29.85
32.03
32.59
36.46
38.0

Fig 2

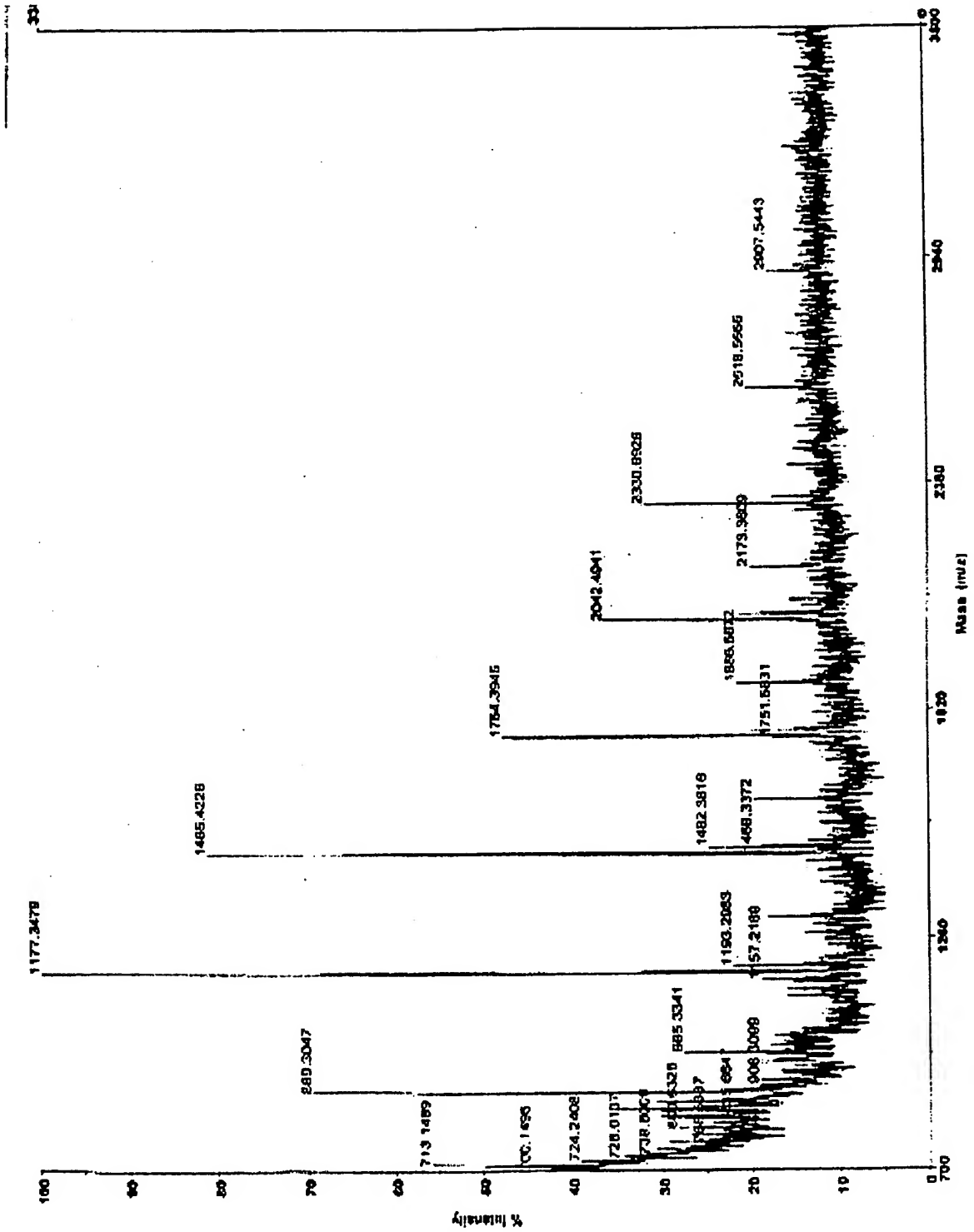

1177.3479
1485.4228
1784.3945
2042.4941
2330.8928
2618.5565
2907.5443
889.3047
1193.2083
1482.3816
1751.5831
2179.3829
% Intensity
Mass (m/z)

Fig 3

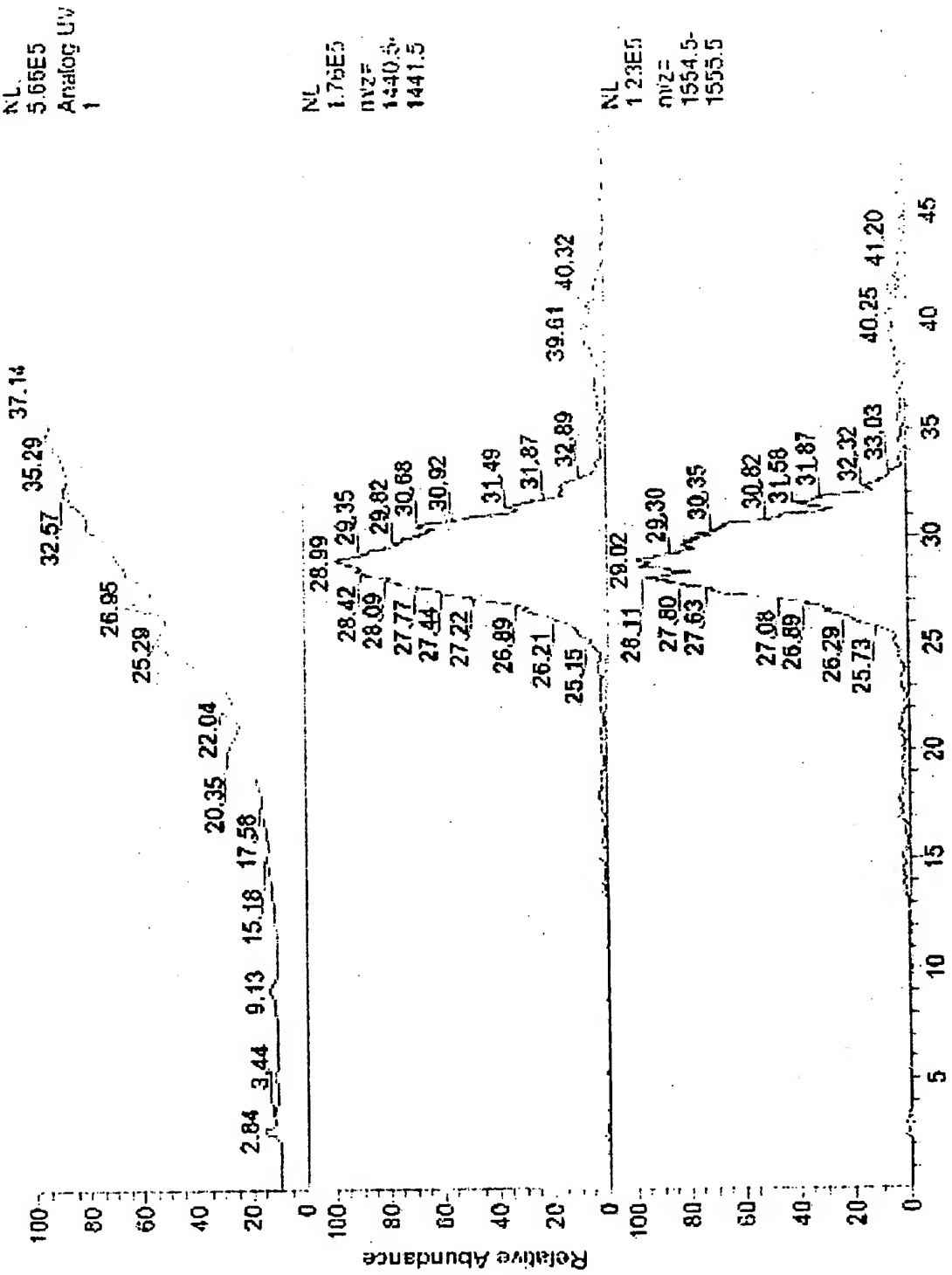

NL
5.65E5
Analog UV
1
NL
1.76E5
m/z=
1440.5-
1441.5
NL
1.23E5
m/z=
1554.5-
1555.5
Relative Abundance
2.84
3.44
9.13
15.18
17.58
20.35
22.04
25.29
26.95
32.57
35.29
37.14
25.15
26.21
26.89
27.22
27.44
27.77
28.09
28.42
28.99
29.35
29.82
30.68
30.92
31.49
31.87
32.89
39.61
40.32
25.73
26.29
26.89
27.08
27.63
27.80
28.11
29.02
29.30
30.35
30.82
31.58
31.87
32.32
33.03
40.25
41.20

Fig 4

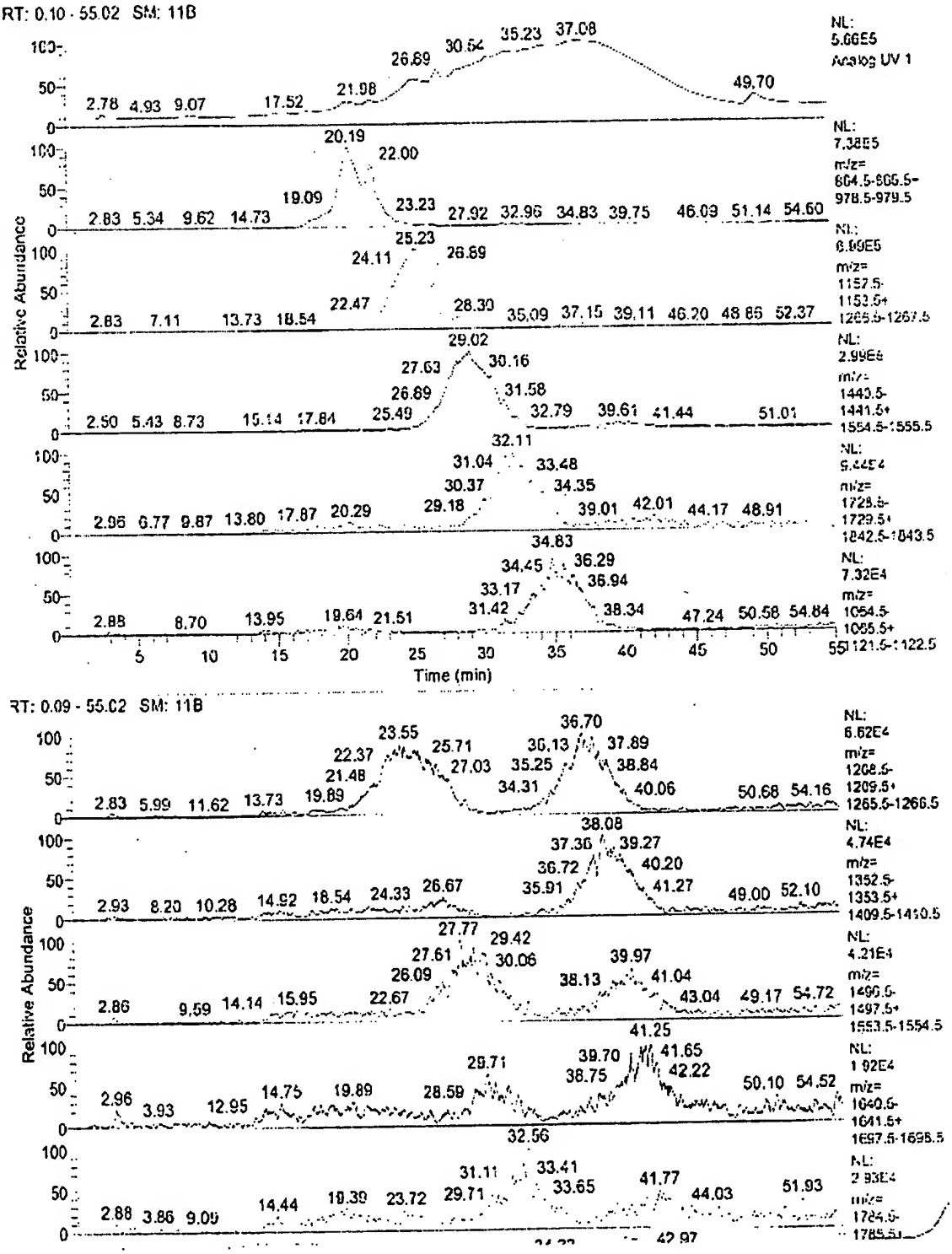

RT: 0.10 - 55.02 SM: 11B
Relative Abundance
Time (min)
RT: 0.09 - 55.02 SM: 11B
Relative Abundance

Fig 5

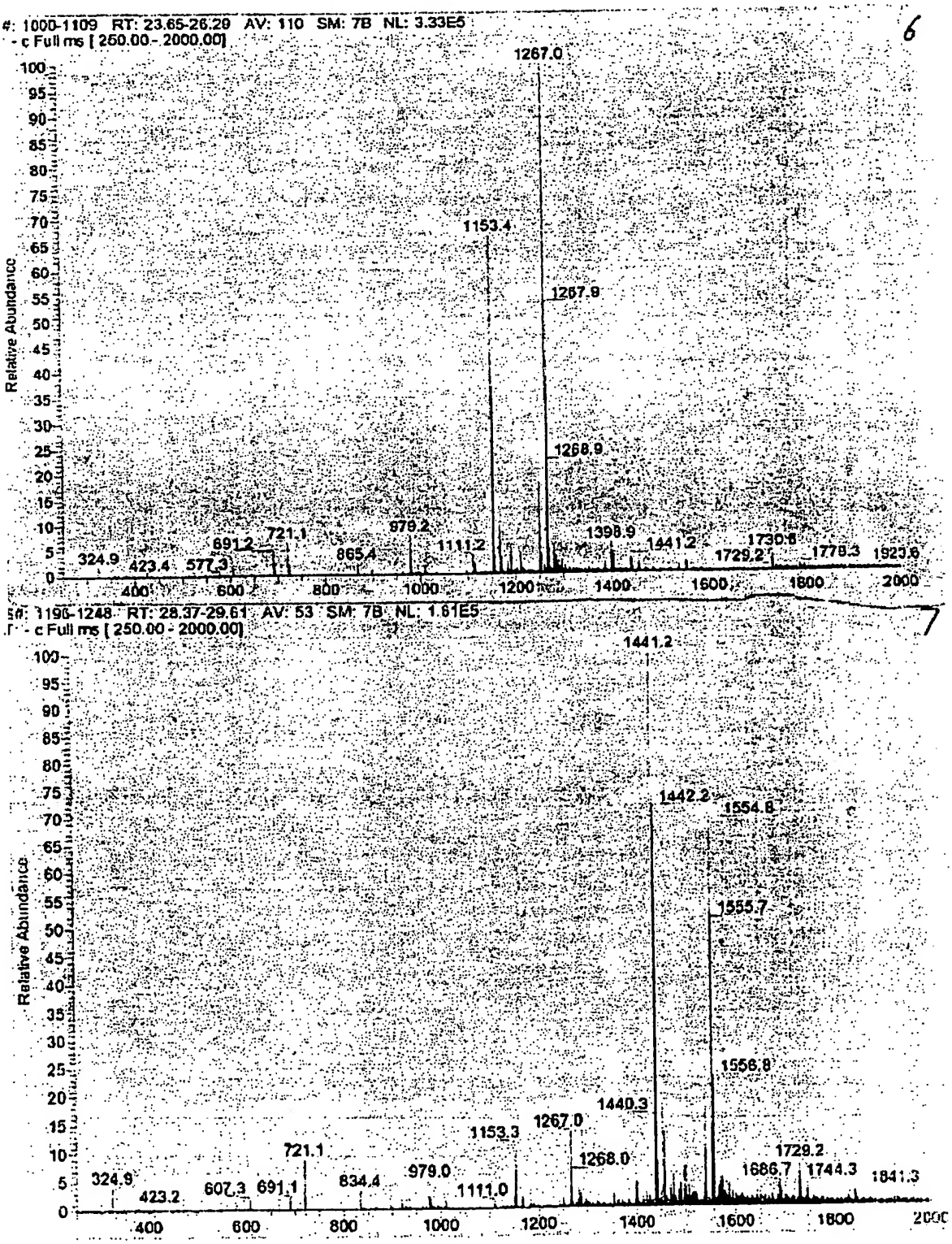
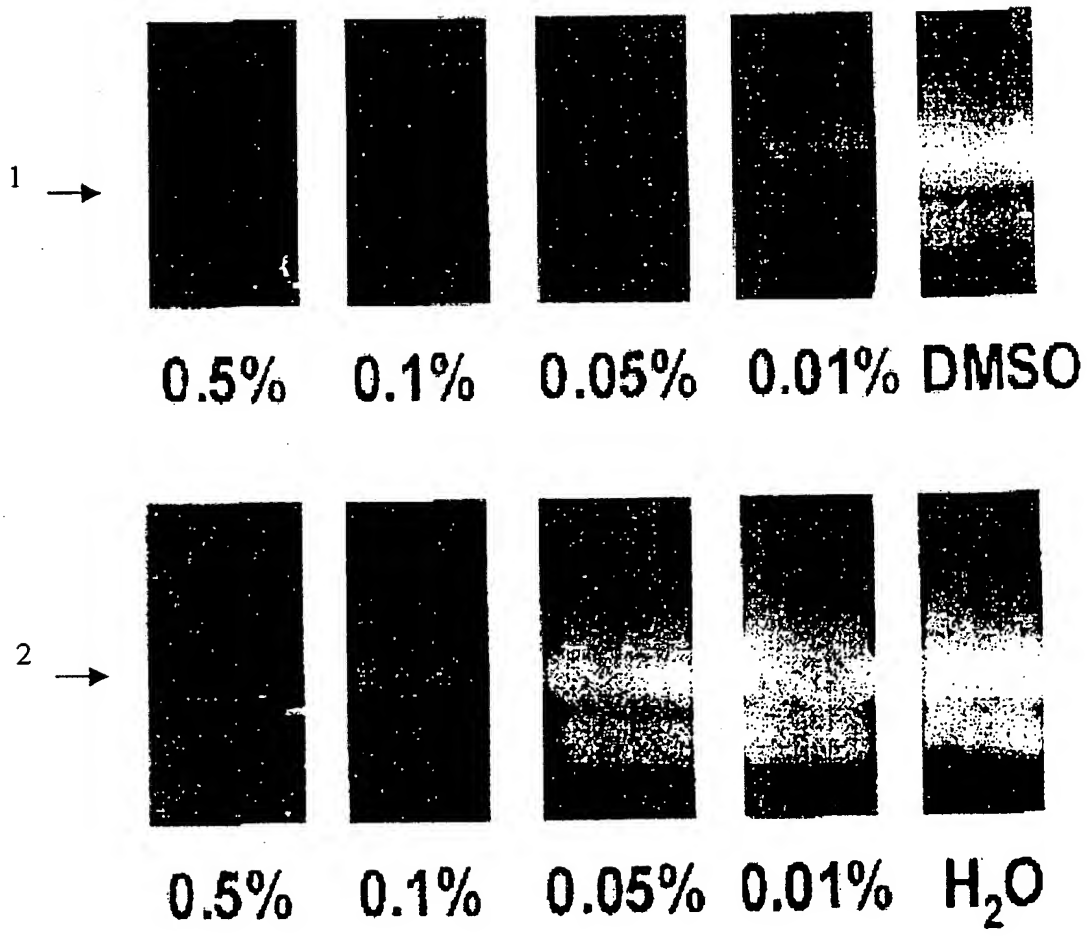

#: 1000-1109 RT: 23.65-26.29 AV: 110 SM: 7B NL: 3.33E5
- c Full ms [250.00 - 2000.00]
6
Relative Abundance
1267.0
1153.4
1267.9
1268.9
879.2
721.1
691.2
865.4
1111.2
1398.9
1441.2
1730.8
1729.2
1779.3
1920.6
324.9
423.4
577.3
#: 1196-1248 RT: 28.37-29.61 AV: 53 SM: 7B NL: 1.61E5
- c Full ms [250.00 - 2000.00]
7
Relative Abundance
1441.2
1442.2
1554.8
1555.7
1556.8
1440.3
1267.0
1153.3
1268.0
721.1
979.0
834.4
1111.0
1729.2
1686.7
1744.3
1841.3
324.9
423.2
607.3
691.1
400
600
800
1000
1200
1400
1600
1800
2000

Fig 6



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR00/00769

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 A61K 31/35

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)

IPC 7 : A61K

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)

CA On-Line, Medline, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	US, A, 5,891,905 (Romanczyk et al.) 06 Apr. 1999, see colum 2, line 35 - line 54 entire document	1, 2, 6-11, 15 3-5, 12-14
X A	JP, A, 08-205,818 (Nonogawa Shoji KK.) 13 Aug. 1996, see page 2, line 12-line 29 entire document	7, 8 1-5, 6, 9-14
A	US, A, 5,646,178 (Walker et al.) 08 Jul. 1997, see entire document	1-15
A	EP, A, 348,781 (Tecnofarmacl S.p.A.) 03 Jan. 1990, see entire document	1-15
A	Ata N et al.'Inhibition by galloylglucose (GG6-10) of tumor invasion through extracellular matrix and gelatinase-mediated degradation of type IV collagens by metastatic tumor cells', In : Oncol. Res., 1996, Vol.8, No.12, page 503-511	1-15
X	Robert L. et al. 'Action of procyanidol oligomers on vascular permeability study by quantitative morphology' In : Pathol. Biol., 1990, Vol.38, No. 6, page 608-616	1, 2, 6, 7, 9

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevence

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 OCTOBER 2000 (27.10.2000)	30 OCTOBER 2000 (30.10.2000)

Name and mailing address of the ISA/KR	Authorized officer
Korean Industrial Property Office Government Complex-Taejon, Dunsan-dong, So-ku, Taejon Metropolitan City 302-701, Republic of Korea Facsimile No. 82-42-472-7140	LEE, Yu Hyung Telephone No. 82-42-481-5603

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR00/00769

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5646178 A	08. 07. 1997	WO 9630033 A	03. 10. 1996
		EP 814825 A	07. 01. 1998
EP 348781 A	03. 01. 1990	US 5484594 A	16. 01. 1996
		JP 2048593 A	19. 02. 1990

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 January 2001 (25.01.2001)

PCT

(10) International Publication Number
WO 01/05397 A1

(51) International Patent Classification[7]: A61K 31/35

(21) International Application Number: PCT/KR00/00769

(22) International Filing Date: 14 July 2000 (14.07.2000)

(25) Filing Language: Korean

(26) Publication Language: English

(30) Priority Data:
1999/28877 16 July 1999 (16.07.1999) KR

(71) Applicant *(for all designated States except US)*: **LG CHEMICAL LTD.** [KR/KR]; LG Twin Tower, Yoido-dong 20, Yongdungpo-ku, Seoul 150-721 (KR).

(72) Inventors; and

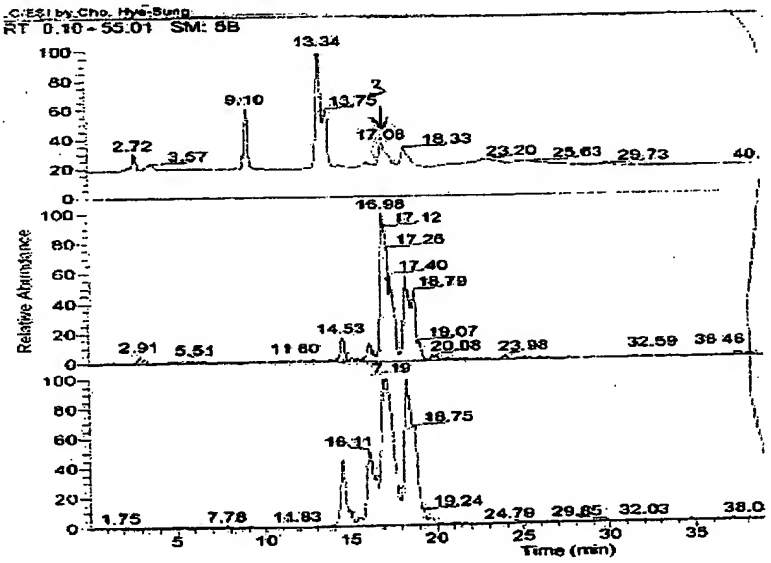
(75) Inventors/Applicants *(for US only)*: **KIM, Sang-Nyun** [KR/KR]; Sejong Apt. 106-102, Jeonmin-dong 462-5, Yuseong-ku, Taejeon-city 305-728 (KR). **AHN, Ho-Jeong** [KR/KR]; Sejong Apt. 107-1106, Jeonmin-dong 460-5, Yuseong-ku, Taejeon-city 305-728 (KR). **PARK, Sang-Ki** [KR/KR]; LG Sataek 2-404, Doryong-dong 386-4, Yuseong-ku, Taejeon-city 305-340 (KR). **KIM, Moon-Moo** [KR/KR]; Lucky Apt. 1-202, Doryong-dong 386-4, Yuseong-ku, Taejeon-city 305-340 (KR). **KIM, Jung-Hun** [KR/KR]; LG Apt. 1-103, Doryong-dong, Yuseong-ku, Taejeon-city 305-340 (KR). **LEE, Hak-Mo** [KR/KR]; LG Apt. 2-404, Doryong-dong, Yuseong-ku, Taejeon-city 305-340 (KR). **PARK, Hyoung-Kook** [KR/KR]; Cheongsol Apt. 512-705, Songgang-dong 8-2, Yuseong-ku, Taejeon-city 305-752 (KR). **CHO, Hye-Sung** [KR/KR]; Hanvit Apt. 110-1401, Eoeun-dong 99, Yuseong-ku, Taejeon-city 305-755 (KR). **JEOUNG, Seung-Young** [KR/KR]; Kolon Apt. 6-502, Nae-dong 151, Seo-ku, Taejeon-city 302-727 (KR).

(74) Agent: **KIM, Won-Ho**; 7th Fl., Teheran Bd., 825-33, Yoksam-dong, Kangnam-ku, Seoul 135-080 (KR).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,

[Continued on next page]

(54) Title: PROCYANIDIN OLIGOMERS INHIBITING MATRIX METALLOPROTEINASES AND MEDICINE HAVING EFFECTIVE COMPOSITION OF THE SAME



(57) Abstract: The present invention provides procyanidin oligomers with significant biological activity against matrix metalloproteinase (MMP). The procyanidin oligomers can be isolated from the genus Ulmus and other plants and comprise trimeric through dodecameric procyanidin oligomers of flavan-3-ol monomer units. The present invention encompasses methods of using the procyanidin oligomer in treating tumor metastasis or invasion, rheumatoid arthritis, diabetes, corneal, epidermal, and gastric ulceration, skin wrinkling, periodontitis, osteoporosis; and in the promotion of wound and burn healing and other related maladies in which uncontrolled high levels of MMP are thought to play an important role in the malady progress.

WO 01/05397 A1

DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

(48) Date of publication of this corrected version:
5 April 2001

(15) Information about Correction:
see PCT Gazette No. 14/2001 of 5 April 2001, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.